Retrospect, advances and challenges in Chagas disease diagnosis: a comprehensive review

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Summary

Chagas disease, caused by *Trypanosoma cruzi*, affects millions worldwide. The 2030 WHO roadmap aims to eliminate it as a public health concern, emphasising the need for timely diagnosis to enhance treatment access. Current diagnostic algorithms, which rely on multiple tests, have prolonged turnaround times. This proves particularly problematic in resource-limited settings. Addressing this issue necessitates the validation and adoption of innovative tools. We explore recent developments in Chagas disease diagnosis, reviewing historical context and advancements. Despite progress, challenges persist. This article contributes to the understanding of current and future directions in this neglected healthcare area. Parasitological methods are simple but exhibit low sensitivity and require supplementary tests. Molecular methods, with automation potential, allow quantification and higher throughput. Serological tools show good performance but struggle with parasite antigenic diversity. Prioritising point-of-care tests is crucial for widespread accessibility and could offer a strategy to control disease impact. Ultimately, balancing achievements and ongoing obstacles is essential for comprehensive progress.

Keywords: Chagas disease; Diagnosis; Surveillance; Point-of-care; Isothermal amplification; Rapid diagnostic tests

Background

Chagas disease (CD), caused by the protozoan *Trypanosoma cruzi* (*T. cruzi*), poses a significant health threat in Latin America with 6–8 million people infected by the parasite and 75 million at risk of contracting the disease. Due to migratory movements of individuals from endemic areas, in the last decade CD has been recorded in several non-endemic countries in Europe, as well as in the USA, Australia, Japan, and other countries.[1,2] CD is a vector-borne disease transmitted by blood-sucking triatomines.[3] There are several additional modes of transmission such as congenital transmission, contact with contaminated meals, and blood/organ exchanges.[4] Despite its widespread impact, CD is classified by the World Health Organization (WHO) as a Neglected Tropical Disease (NTD),[5] emphasising the urgent need for increased attention and resources. Untreated, up to 30% of infected individuals may develop life-threatening cardiac and/or chronic digestive diseases.[6] Alarmingly, only 3% of *T. cruzi* carriers are diagnosed, with just 1% undergoing treatment.[7,8]

In 2010, the 63rd World Health Assembly urged governments to establish and apply algorithms for NTDs diagnosis. Accurate and timely detection of *T. cruzi* infections is key to improving treatment access. This article examines dynamic developments in CD diagnosis and reviews the historical context and recent advancements. It explores progress in the identification and confirmation of the infection with insight into cutting-edge methodologies. Despite these advancements, persistent challenges remain. This review aims to contribute to the improvement of CD diagnosis by fostering a deeper understanding of the current state and future directions in this neglected healthcare area.

Phases of disease and diagnosis

The stage of the disease and the transmission pathway dictate the appropriate diagnostic approaches (Fig. 1). The acute phase of the infection often goes unnoticed, with 95% of cases being asymptomatic. Additionally, it may manifest as a self-limited febrile illness which can be difficult to distinguish from other febrile illnesses. Without antiparasitic therapy, newborns with congenital

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infection, children, and immunocompromised individuals face an increased risk of severe symptoms after an incubation period of seven to 14 days.[9] In endemic areas, acute cases can also result from classical vector transmission by which infected triatomines (referred to as kissing bugs) defecate the parasites on the skin during or after a blood meal; reactivation in immunocompromised individuals, such as those co-infected with HIV and organ transplant recipients; and oral transmission.[10] Notably, orally-acquired *T. cruzi* infections are more severe, with higher parasitic loads and mortality rates, than infections from the classical vector-borne transmission route.[11]

In both endemic and non-endemic settings, acute cases of CD can arise through non-vectorial pathways. Particularly significant is congenital transmission, where neonates born to *T. cruzi*-infected mothers are affected. This mode of transmission, prevalent outside endemic regions, significantly contributes to the urbanisation of the disease. Congenitally infected infants may exhibit severe manifestations, but the majority remain asymptomatic. Consequently, diagnosis and treatment are unlikely unless actively sought.[12]

Detecting vertically acquired *T. cruzi* infections in neonates is crucial because of the potential for the development of severe disease and the risk of perpetuating vertical transmission when infected girls reach reproductive age. This underscores the importance of targeted screening efforts to identify and address congenital CD cases early when available antiparasitic drugs are almost 100% efficacious and well-tolerated.

Transmission through the transfusion of blood or blood derivatives,[13] or through organ transplantation,[14] are also relevant in endemic and non-endemic regions. The resulting acute cases can have high parasitaemia levels. Hemovigilance for CD has become systematic in many endemic and non-endemic countries. For example, countries like the USA, UK, Spain, France, Switzerland, and Portugal have adopted the screening of blood products from individuals with epidemiological risk factors for *T. cruzi* infection.[13] After transplantation, monitoring recipients of organs from *T. cruzi*-infected donors is crucial to detect the emergence of bloodstream trypomastigotes due to immunosuppressive conditions.[15] Solid organ transplant recipients with chronic CD who experience immunosuppression, through immunosuppressive drugs[13] or autoimmune diseases or cancer, are also prone to reactivation.[15]

Left untreated, the acute phase can develop in multiple ways. A chronic asymptomatic infection occurs in 70% of cases. This can persist throughout life. In the remaining 30% of cases, development of symptoms occurs within 10–30 years, leading to a chronic symptomatic infection. This can affect various organs including the heart (20–30% of cases), gastrointestinal tract tissues (15–20% of cases), and the peripheral nervous system (sensory polyneuropathy, less than 5% of cases).[16] Cardiac myocardiopathy stands out as the most prevalent manifestation in the chronic symptomatic phase.

The chronic phase is marked by low parasitaemia, and diagnosis relies on patients' immunological responses. As depicted in Fig. 1, there is a short temporal gap between the conclusion of the acute phase and the emergence of elevated antibodies in the chronic phase. This interval poses a challenge for several existing diagnostic approaches. Enhancing the sensitivity of

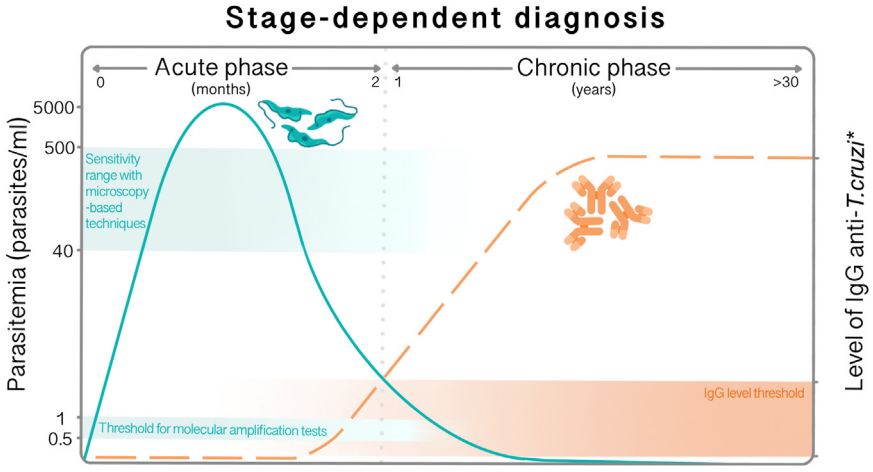

***Fig. 1:* Chagas disease stage-dependent diagnosis**. *Anti-*T. cruzi* IgG levels can be expressed as serum dilutions in hemagglutination inhibition assays (HAI) output or as optical density (OD) reactivity values (arbitrary units) in enzyme-linked immunosorbent assays (ELISAs). In both instances, a positivity threshold of 1/16 or approximately 0.2 is applied for HAI and ELISA, respectively. This means that results equal to or exceeding these thresholds are considered positive for anti-*T. cruzi* IgG.

current molecular methods and advancing immunological techniques for early antibody detection could strategically bridge this gap, thereby enhancing diagnostic efficacy during the transition between infection phases. In this context, serological assays targeting anti-*T. cruzi* type M circulating immunoglobulins (IgM) have been suggested, primarily for their potential to detect recently acquired infections specifically. However, the non-specific reactivity associated with IgM's large size underscores the preference for IgG-targeted tests.

The impact of *T. cruzi* diversity and clonal histotropism in diagnostics

For several years, various genetic structures and nomenclature have been proposed for *T. cruzi* classification. Currently, there are seven accepted lineages termed discrete typing units (DTUs): TcI–TcVI and TcBat, a newer lineage isolated from bats. DTUs describe sets of parasites that are genetically more similar to each other than to other stocks and can be identified by common genetic markers.[17] They are genetically diverse with distinct geographical distribution, pathogenicity levels, and susceptibility to antiparasitic treatment.[18]

TcV and TcVI arose from hybridisation events of TcII and TcIII. While Tc III and TcIV are mostly associated with the parasite wildlife cycle, genotypes TcI, TcII, TcV, and TcVI have been described to cause pathogenic infections in humans. There is also an increasingly recognised intra-lineage diversity, with DTU TcI subdivided into TcIa-e[19,20]; and TcIV structured into distinct lineages grouping in North and South America.[21] Hence, diagnostic approaches must possess comparable sensitivity and specificity for detecting infection across all parasite variants. This presents a challenge in the development of all serological and molecular tests intended for comprehensive detection coverage.

Studies reveal that *T. cruzi* strains, sourced from both triatomine vectors and vertebrate hosts, are multiclonal. In the chronic infection stage, specific *T. cruzi* subpopulations may undergo selection, displaying differential tissue tropism.[22] Given the parasite's heterogeneity, simultaneous infection by different strains in the same host is possible. The clonal histotrophic model proposes that strain heterogeneity and multiclonality contribute to diverse tissue tropism, resulting in varied clinical presentations.[23] In CD patients, cardiac tissue clones differ from oesophageal clones,[22] and divergent subpopulations have been detected in heart tissue slices from the same transplant patients.[24] Reactivation of parasites in target tissues may contribute to dynamic disease behaviour, observed in immunosuppressed patients as panniculitis, myocarditis, or meningoencephalitis.[24]

Understanding the parasite's life cycle and dormancy is crucial for CD diagnosis and monitoring. In vitro, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assays and 5-ethynyl-2′-deoxyuridine (EdU) labelling reveal replicating and non-replicating amastigotes, differentiating amastigote-to-trypomastigote forms, and non-replicative trypomastigotes coexisting in a single host cell.[25] Parasite replication dynamics within host cells lack a predictable or tightly regulated pattern. This is observed across disease phases or specific infected tissues, both in vitro and in vivo,[25] and potentially influences parasite detection and quantification during infection and treatment monitoring.

Laboratory diagnosis according to disease phase and transmission settings

Laboratory diagnosis relies on parasitological, serological, and molecular techniques. These are applied based on the stage of *T. cruzi* infection and the route of transmission. Table 1 provides a concise overview of the key characteristics and applications of current assays used in diagnosing CD. Moreover, Supplementary Table S1 furnishes definitions for key terms relating to analytical and operational parameters utilized in assessments, validations, or verifications of diagnostic techniques.

Parasitological methods

In the acute phase, *T. cruzi* trypomastigotes can be observed in freshly collected peripheral blood through light microscopy, yielding immediate but time-consuming results (15–30 min per preparation). Historically, several microscopy-based approaches have been used in CD diagnosis. For instance, Giemsa-stained thin and thick blood smears at 500–1000 × magnification were utilised for confirming and morphologically characterising parasites. However, their suboptimal sensitivity, with a limit of detection of ~500 parasites per mL, discourages their use for clinical diagnosis. Blood concentration methods like the Strout or the microhematocrit/micromethod, in which blood is centrifuged and the buffy coat is examined, enhance the probability of observing the parasites.

The Strout, recommended for detecting parasitaemia in adults with suspected acute infection, requires a large sample volume of venous blood (5–10 mL) and it is not recommended for diagnosing neonatal congenital infections or acute infections in children.[29,41] In contrast, the blood drawn for the microhaematocrit or micromethod parasite concentration methods ranges from 0.3 to 0.6 mL and is collected using capillary tubes or microtubes, respectively. Simplicity and cost make microhaematocrit the preferred parasitological test for detecting congenital infection in neonates.[30] Most congenital CD diagnosis guidelines suggest running up to two parasitological tests, one at birth with either

Type of test	Diagnostic use	Reagents storage and transportation	Sample	Limit of detection	Quantitative results	Clinical sensitivity	Clinical specificity	Observations	Refs.
Parasitological									
Fresh blood	Acute CD	NA	5–10 mL peripheral whole blood	>500 par/mL	No	<40%	>98%	Low complexity, but microscopy required; 15–30 min per preparation.	26
Strout	Acute CD	NA	5–10 mL peripheral whole blood	NA	No	<50%	>98%	Low complexity. Involves two centrifugation steps before microscopy. Ideally, it should be done within 2 h of blood collection.	27,28
MH/MM	Acute CD, congenital CD	NA	0.3–0.6 mL whole blood	>50 par/mL	No	50%	>98%	Same as the other parasitological methods, it is microscopy-based, operator dependent, has a low throughput (15–30 min per determination), and the time elapsed between sample collection and examination is critical.	29,30
Molecular									
PCR	a) Acute CD and infection reactivation, congenital CD b) Post-treatment follow-up	Reagents must be transported and stored at -20 °C	a) 1–5 mL whole blood (EDTA or GEB treated), biopsy samples, cerebrospinal fluid b) 1–5 mL whole blood (EDTA anticoagulated)	0.5 to 1 par/mL depending on DTU	Yes, upon including standard curve	70–98% Acute and congenital CD; 50–65% in untreated chronic CD	>98%	rtPCR assays based on satDNA or kDNA sequences. Require expensive equipment and molecular biology level facility, highly trained personnel, and the cost of reagents is high (single determination 35–70 USD).	31–33
LAMP	Acute CD, including congenital CD	Room temperature, no cold chain required	a) 30 µL liquid whole blood anticoagulated with heparine b) Single 3–6 mm punch of whole blood DBS in filter paper	a) 1 to 5 par/mL depending on DTU b) 10 to 20 par/mL depending on DTU	No	93–97%	>94%	LAMP assay based on satDNA sequence. POC test, no major or expensive equipment required. Feasible at low-resource settings. ASSURED compliance. Still need to be validated as a tool for treatment follow-up. Estimate cost range 8–12 USD per determination.	34,35
Serological[a]									
HAI	Congenital CD > 9 months Chronic CD	Refrigerated	Serum	NA	Yes	73–99%	60–97%	Lower cost and no need of equipment make of HAI a first diagnostic option in vast areas endemic to CD. But poorer performance than ELISA o IIF assays described.	36
ELISA	Congenital CD > 9 months Chronic CD	Refrigerated	Serum or plasma	NA	Yes	28–99%	>96%	Better to use spectrophometer for read out. Generally have a high performance with Se/Sp > 95%; in regions like Bolivia the agreement between ELISAs is nearly perfect, but in Central America and Mexico Se is <80%. Market cost per determination <3 USD.	36,37
IIF	Congenital CD > 9 months Chronic CD	Refrigerated	Serum or plasma	NA	Yes	NA[b]	NA[b]	Low throughput. Requires of fluorescent microscope and trained personnel. Used as tiebreaker test in reference laboratories; it is labor-intensive and often based on in-house protocols.	-
CMIA	Congenital CD > 9 months Chronic CD Blood bank screening	Refrigerated	Serum or plasma	NA	Yes	>99%	>99%	High throughput capacity, operator-independence, and automated functionality make it highly suitable for blood bank screening. High cost of equipment and reagents limits its use to centralized blood banks and high-resource settings.	38

(Table 1 continues on next page)

Type of test	Diagnostic use	Reagents storage and transportation	Sample	Limit of detection	Quantitative results	Clinical sensitivity	Clinical specificity	Observations	Refs.
(Continued from previous page)									
RDTs	Congenital CD > 9 months Chronic CD	No cold chain	5–100 µL whole blood finger-pricked	NA	No	27–99%	87–97%	POC tests with results turnaround within 1 h; easy-to-use and no equipment required. Performance feasible at low-resource settings (ASSURED compliance), and market costs ranging 2 to 8 USD per single test.	37,39,40

CD, Chagas disease; CMIA, chemiluminescent microparticle immunoassay test; DBS, dried blood spot; DTUs, discrete typing units; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; GEB, guanidine-EDTA-blood; IIF, indirect immunofluorescence assay; HAI, hemagglutination inhibition assay; kDNA, kinetoplast DNA; LAMP, loop-mediated isothermal amplification; MH/MM: Microhaematocrite/Micromethod, NA, not applicable; par/mL, parasite equivalents per mL of sample; PCR, polymerase chain reaction; RDT, rapid diagnostic test; rtPCR, real time PCR; satDNA, satellite DNA; Se, sensitivity; Sp, specificity. [a]Serological tools have a geographic dependent performance due to the wide genetic and antigenic variability of *T. cruzi*. The use of RDTs is acknowledged in the guidelines for the management of chronic and congenital Chagas disease cases from Paraguay (https://senepa.gov.py/materiales/). [b]Clinical sensitivity and specificity not applicable to IIF since this is a confirmatory test.

Table 1: **Overview of key characteristics and applications of current parasitological, serological, and molecular assays used in diagnosing chagas disease.**

venous or umbilical cord blood, and another during the first months of life. Microhaematocrit and micromethod with limited analytical sensitivity (40–50 parasites/mL; Fig. 1), rely on trained operators.[42] Although a positive result offers an unequivocal diagnosis and enables immediate treatment, the test's average 50% clinical sensitivity, compared with delayed serology,[43] is far from optimal and requires a confirmatory serological study at 8–12 months when maternal antibodies wane.[42] At this point, a positive serological result conclusively infers congenital infection in infants not diagnosed by parasitological methods. Such a long-spanning algorithm, involving up to three tests, is not practical as it creates a high risk of loss to follow-up. Moreover, microscopy-based parasitological diagnosis should ideally occur within 2 h of blood collection to ensure live trypomastigote detection. As more time elapses between blood collection and examination, sensitivity decreases. Thus, the necessity for a quick turnaround time is a major drawback.

Other indirect parasitological detection methods include xenodiagnosis,[44] hemoculture,[27,45] or animal inoculation.[46] Each depends on an intermediary step that can prolong the turnaround of results by several days or weeks: feeding laboratory-raised triatomine insects on the suspected patient, culturing the subject's blood, or injecting it into mice to amplify the potentially present trypomastigotes, respectively. Due to biosafety concerns, labour intensiveness, and the long delay in producing results, these methods are rarely used at present.

Serological methods

During the chronic phase of infection, parasitaemia is low and intermittent, reducing the sensitivity of direct parasitological techniques.[47] Consequently, methods for detecting antibodies against *T. cruzi* are much more useful in diagnosing the infection (Fig. 1). However, because of the ample antigenic diversity of *T. cruzi*, the current diagnosis algorithm recommends the agreement of two tests based on distinct antigenic principles for a confirmed positive or negative output. If discordant, a third test should be done.[26,28]

Because of their generally high sensitivity and specificity, the most used serological methods are enzyme-linked immunosorbent assays (ELISAs). In comparison to indirect immunofluorescence (IIF), another immuno-assay, ELISAs have a simpler protocol and require a spectrophotometer, rather than a fluorescence microscope. ELISAs are based on total whole parasite lysates, purified parasite antigenic fractions, or recombinant proteins. The ELISAs based on recombinant antigens were developed seeking better reproducibility and reliability due to standardisation of reagents production.[48,49] Nonetheless, requiring the concordance of two tests with distinct antigens to yield a diagnosis often means a lysate-based and a recombinant-based ELISA are needed. In many laboratories, the hemagglutination inhibition assay (HAI), which is based on the ability of anti-*T. cruzi* IgG to agglutinate a suspension of sheep red blood cells sensitised with antigens from the parasite, substitutes for one of the ELISAs. The cost of the HAI is less than that of the ELISA or IIF, but its sensitivity is also lower. ELISAs, IIF, and HAI are considered conventional serological tools, and there are several available for the detection of chronic *T. cruzi* infection.[36,50,51] In any case, the fact that two tests, and sometimes a third, are needed results in a delayed turnaround of results and the subsequent risk of loss to follow-up. Moreover, these conventional tools use serum or plasma, which must be segregated from a volume of blood between 1 and 5 mL extracted by venipuncture. In addition, the sera or plasma samples and some of the reagents of the kits require refrigeration, restricting their use to equipped laboratories. In response to these disadvantages, easy-to-use rapid diagnostic tests (RDTs)

based on immunochromatographic lateral flow detection of anti-*T. cruzi* IgG were developed.[52] Many of them can use small volumes (between 5 and 100 μL) of whole blood collected via finger-prick as samples, which, together with their equipment-free quick turnaround of results (often less than 30 min), and their working and storage at room temperatures, make them point-of-care (POC) tests. For further details of the 25 commercially available CD RDTs we refer the readers to the recent review by Gabaldón and collaborators.[52]

RDTs' POC advantages prompted investigators to evaluate the use of a combination of these tests as an alternative to conventional tools for detecting chronically infected subjects in Bolivia,[39] Argentina,[53] and Colombia,[54] with very good performance agreements to the ELISAs or inter-RDTs. The collected evidence would support their use for confirmatory diagnosis in those areas.

Nonetheless, variations in the performance of conventional, recombinant-based serological tests and RDTs have been reported between regions and countries.[55] Particularly in Mexico, they have demonstrated alarmingly poor performance with sensitivities no greater than 30%.[37] This is likely due to the high genetic and antigenic diversity of the parasite in this region. Notably, about 25% of the proteins expressed by *T. cruzi* are found in tandems of 5–68 amino acids.[56] Some *T. cruzi* strains circulating in different geographical areas contain only a limited number of antigenic determinants that are not expressed, either partially or at all.

An evaluation of tests' performance among blood donors in the USA indicated that for all tests evaluated, antibody reactivity and clinical sensitivity were lowest in donors from Mexico, intermediate in those from Central America, and highest in those from South America. However, the minimum sensitivity reached at least 82.6%.[37,57] In another study analysing the reactivity of two RDTs and an ELISA in maternal blood samples from Argentina, Honduras, and Mexico, the overall reactivity of the three tests was relatively low and there were significant differences among countries. More than 12% of cases of *T. cruzi* infection in Argentina, more than 21% in Honduras, and as many as 72% in Mexico were not detected.[37] Besides the differences in the *T. cruzi* strains predominantly circulating in each country,[58] it is likely that genetic differences between human populations may contribute to discrepancies in the serology outcome.[59] Hence, *T. cruzi* antigenic diversity endorses the need to pre-validate the serological tools that are to be used in a given region, be it conventional or RDTs.[60]

Therefore, in scenarios where RDTs performance is equivalent to that of the conventional tests, their use for confirmatory diagnosis should be considered. Furthermore, if two conventional tests or RDTs, based on distinct antigenic principles, have a very high level of agreement (Cohen's Kappa test >0.80) when evaluated on a certain region, the use of a single test should be considered.[61,62] Such an approach may be unfeasible in regions where improvement in serological diagnoses is needed to ensure optimal case identification. However, in other areas where evidence of their high-level performance already exists, this approach could greatly contribute to saving costs. Although the market price of a serological RDT may be more costly than a conventional serological method, their POC attributes place them in an advantageous position over the latter, especially for their use in areas without well-equipped laboratories.[39] A cost-effectiveness analysis comparing them to conventional tools, which takes into account issues like time worked by laboratory professionals and the risk of loss to follow-up is yet pending.[63]

In addition to ELISAs and RDTs based on recombinant proteins or multi-epitope peptide sequences as antigens, other innovative immunodiagnostics incorporate recombinant antigens. Examples include the ARCHITECT assay and the Elecsys system.[34,38,64] Reagents for detecting CD using these platforms were developed in recent years, with the goal of developing a single assay. These systems have been described to yield very high performances, have chemiluminescent readouts and automation potential, and allow a much higher throughput.[34] However, they are very costly pieces of equipment that require robust laboratory facilities and highly trained personnel, which largely limits their use. At present, they are available in the laboratories of large hospitals, mostly in non-endemic countries, and in central blood bank screening facilities. There, the high sensitivity, automation, and high throughput rendered by these platforms are paramount.[40]

Novel approaches to develop improved serological assays

Several studies have demonstrated that *T. cruzi* antigens used in current tests and their strain-variants and epitopes thought to be broadly antigenic failed to be recognised by serologically discordant samples from different regions in the Americas.[37,40] Hence, the identification of novel diagnostic antigens must be pursued. New approaches, including the use of high-density peptide microarrays, are providing powerful opportunities to screen a large number of antigens, allowing for the identification of many new antigenic entities recognised by well-characterized sera, including discordant samples.[65–67] At the genetic level, next-generation sequencing (NGS) of underrepresented parasite genomes, and whole genome sequencing analysis, will grant the identification of highly conserved protein-coding sequences among isolates from all DTUs and geographic distributions. As a result, it may be possible to find "universal" antigens for the design of improved diagnostics. These studies are critical for the development of regionally tailored or unique ELISAs and RDTs suitable to be used across the Americas.[60]

Molecular methods

In recent decades, molecular diagnosis for *T. cruzi* infection has gained prominence, particularly in critical applications such as early detection of congenital transmission, diagnosing food-borne transmissions, identifying infections in organ recipients from CD donors, monitoring reactivation in immune-suppressed patients, and assessing treatment response.[68] However, according to a recent systematic review and meta-analysis, PCR demonstrates low sensitivity in the chronic phase (67%, 95% CI 65.4–68.5).[68] This limitation may be attributed to insufficient concentrations of parasitic DNA in blood samples from chronically infected patients, falling below the detection limits of the currently available methods. Collecting serial samples or increasing blood volume may address this limitation.[68,69]

Polymerase chain reaction

Real-time PCR (rtPCR) has exhibited acceptable performance for early *T. cruzi* detection and quantification of parasitic loads in scenarios like vertical[70,71] and orally-acquired *T. cruzi* transmission,[72] primary infection after transplantation with contaminated donor organs,[73] and immunosuppression-induced reactivation.[74] While PCR demonstrates over 90% sensitivity during the acute phase, its utility in the chronic phase is debated due to variable sensitivities and fluctuating parasitaemia.[75–77] Influencing factors include sample volume, DNA extraction method, and PCR target.[31–33] Commercial rtPCR tests recently developed and registered as *in-vitro* diagnostics (IVDs) adhere to an international consensus promoted by WHO-TDR to standardize PCR techniques.[78] Real-time PCR offers advantages like automation, sample preservation, organized laboratory routines, and support for external quality control programs.[76,79] It currently stands as the gold standard for molecular disease diagnosis due to its robust evidence base, notable sensitivity and specificity, quantification capacity for monitoring CD, and regulatory, agency-approved commercial kits.[79–81] The molecular targets with the highest sensitivities and specificities are the conserved regions of kinetoplastid DNA minicircles and satellite DNA repeat sequences. This is due to their high copy numbers, although variations in analytical sensitivity may differ according to the gene dosage of circulating strains.[78]

Despite its absence in the Pan-American Health Organization (PAHO) CD diagnostic recommendations, some endemic countries like Argentina and Chile have incorporated rtPCR into patient care guidelines as an alternative to direct parasitological tests. The wide use of these highly sensitive techniques is crucial in PAHO's initiative for the elimination of mother-to-child transmission of HIV, syphilis, hepatitis B, and Chagas disease (EMTCT-plus). This initiative aims to diagnose and treat at least 90% of vertically infected children in the coming years, with the eventual goal of eliminating mother-to-child transmitted pathogens in the Americas.[28] Although PCR is more effective than parasitological methods in diagnosing acute and congenital *T. cruzi* infections,[19] it is constrained to reference centers or research settings. This limitation results in its unavailability in many endemic areas.

Loop mediated isothermal amplification

Loop-Mediated Isothermal Amplification (LAMP),[82] which demonstrates comparable sensitivity, inclusivity and specificity to PCR, has a simpler, more cost-effective infrastructure. It outperforms PCR in ease of use and detection of *T. cruzi* in very low sample volumes (30–200 µL), thus making it a crucial diagnostic tool for newborns with congenital infections in endemic and non-endemic regions.[34,82] The integration of LAMP into POC diagnostics represents a significant advancement in the pursuit of faster, more accessible testing.[35] Remarkably, repurposing 3D printers for DNA extraction and ultra-rapid DNA extraction systems addresses practical challenges, thus enhancing LAMP feasibility in field settings.[83,84] Its operational use aims to streamline diagnostic processes, thereby improving accessibility, particularly in resource-limited environments lacking traditional laboratory infrastructure. Moreover, LAMP, compatible with anticoagulated liquid blood has demonstrated efficacy with dried blood spots (DBS). These DBS can be conveniently stored and transported at room temperature from peripheral sites, which could be a major factor in the widespread acceptance of the technology.[84]

Recombinase polymerase amplification

The RPA-LF (lateral flow) system, though not yet assessed in patients, effectively identified *T. cruzi*-infected dogs in an endemic area in Mexico.[85] Utilising retrospective samples from dogs with *T. cruzi* infection confirmed through ELISA, Western blot, and quantitative rtPCR, the RPA-LF system amplified *T. cruzi* DNA at concentrations of 1–2 parasites per reaction. It exhibited 95% repeatability at two parasites per reaction, demonstrated no cross-reactivity with human DNA or other protozoan parasites, and encompassed all DTUs. With a sensitivity of 93.2% (95% CI 87.2–98.1) and excellent agreement with PCR (Cohen's Kappa test = 0.963), the diagnostic efficacy of the RPA-LF system justifies extensive field-testing in endemic areas. However, this recommendation is contingent upon its validation in human samples.[85]

Polymerase spiral reaction

Polymerase spiral reaction (PSR) presents an innovative approach for on-site diagnosis of concealed infections. A recent study successfully optimized colorimetric-based PSR for the detection of *Trypanosoma evansi* by

Setting	Recommended testing strategy	Strengths	Limitations	Observations	Refs.
Epidemiological surveys	Rapid Diagnostic Tests (RDTs)	Easy to use, fast turnaround of results, functional with tiny volume of whole blood collected by finger prick, no need for cold chain or equipment, and can be implemented even outdoors.	The widespread use of RDTs throughout all endemic areas is still limited due to the heterogenous immune response of patients from different regions and parasite diversity. The use of a conventional serological test is needed for confirmatory diagnosis.	They have been shown to perform better in high-prevalence settings/ populations. Anyhow, preliminary testing of the RDTs to use at large in a certain region is highly advisable. Similarly, as for other serological assays (i.e., conventional ones), due to the wide antigenic diversity of the parasite.	37,52–54,56,57,60,61,63
Blood/organ donor screening	CMIA automated serological tests.	They yield a very high sensitivity and a high throughput.	The cost of CMIA assays and equipments is high, meaning they are only available in central reference laboratories.	WHO recommends to use at least two assays with different principles to confirm a positive serological result for *T. cruzi* infection. Generally, ELISA is used to confirm.	49
Screening of women at childbearing age and/or pregnant to prevent vertical transmission (e.g., in the context of WHO/PAHO's EMTCT-plus initiative).	RDTs, if no former serological outcome is available and screening must be made bedside close to the delivery; any serological assay if the screening is performed during the pregnancy follow-up visits.	In the case of women at childbearing age it allows access to confirmatory diagnosis and treatment, which will prevent vertical transmission and the potential development of symptomatology in the women. In the case of pregnant women it allows appropriate study and follow-up of offspring at birth, and access to confirmatory diagnosis to receive treatment upon completion of the breastfeeding period.	Use of repeated serologic assays, including ELISA, IIF, and/or HAI. WHO recommends to use at least two assays with different principles to confirm a positive serological result for *T. cruzi* infection.	–	62
Clinical diagnosis					
Acute phase	Use of thick smear, Strout or equivalent parasitological techniques or Real Time PCR (RT-PCR).	Molecular methods allow rapid and sensitive detection of acute stage infections. They outperform parasitological methods thanks to their higher sensitivity.	Parasitological methods are operator dependent and lack sensitivity. PCR is costly and requires of trained personnel and well-equiped laboratories.	Ig M based assays are not routinely used. Validation studies suggest the LAMP could be used as a POC molecular test, although more studies in other territories (e.g., Central and Northern America) are still needed. Evidence to validate it in the field for vectorial and orally acquired acute infections is also pending.	11,12,29,30,34,35, 41–43,64,70,71,81,83,84
Congenital CD (vertical infections)	Algorithm involves the use of micromethod/ microhematocrite or RT-PCR close to delivery and/or around one month of life. In case of non-detectable parasitological and/or molecular findings, the infant must be followed-up for serological diagnosis after nine months of life.	Molecular methods allow rapid and sensitive early detection of vertically acquired *T. cruzi* infection and outperfom parasitological methods, diminishing the loss to follow-up particularly observed in vulnerable populations.			
Chronic phase	Use of repeated serologic assays, including ELISA, IIF, and/or HAI. WHO recommends to use at least two assays with different principles to confirm a positive serological result for *T. cruzi* infection	In many regions, the algorithm for serological detection using ELISA, CMIA is highly sensitive, which together with a highly specific method such as ELISA or IIF are most desired to confirm infection.	Long turnaround times, compromising the access of patients to treatment. Highly sensitive methods can present false positive results.	There exist a variety of antigens, such as total *T. cruzi* antigens, purified total antigens, recombinant proteins or synthetic proteins or peptides.	36,38,40,50,51

(Table 2 continues on next page)

Setting	Recommended testing strategy	Strengths	Limitations	Observations	Refs.
(Continued from previous page)					
Immunosuppression conditions in chronically infected subjects leading to infection relapse (AIDS, organ transplantation in receptor positive individuals), **or receptors of organs from *T. cruzi*-infected donors.**	Use of thick smear, Strout or equivalent parasitological techniques, RT-PCR.	RT-PCR renders a timely detection of *T. cruzi* primary infection in bloodstream. It also allows for the earlier detection of infection reactivation in skin chagomas or endomyocardial biopsies in cases of heart transplant. Increase of parasitic loads by PCR after transplantation allows for earlier assumption of infection reactivation with respect to Strout.	RT-PCR cost and the need of highly trained personnel and well-equipped laboratories.	RT-PCR can be quantitative given the appropriate standard curve is included.	10,15,24
Evaluation of treatment effects					
In clinical trials	Use of bloodstream-based quantitative RT-PCR as surrogate biomarker of treatment failure.	As reduction of serological titres or seroconversion have a long delay, RT-PCR is the currently trusted tool for earlier detection of treatment failure.	In chronic patients, clinical trials that mandate baseline RT-PCR positivity for patient enrollment can impede recruitment speed. Various approaches are employed to address this challenge, including the collection of serial samples or conducting RT-PCR replicates.	RT-PCR negativation does not mean parasite eradication. Dynamics of parasitic load increase after ending treatment and during post-treatment monitoring may be useful to compare efficacy between different treatment arms.	69,77
In healthcare settings	Use of serological markers to address decrease of serological titres, and RT-PCR to detect treatment failure.	Persistence of *T. cruzi* DNA after treatment, detected by parasitological or molecular methods, indicates failure.	In chronic patients, clinical sensitivity of parasitological methods is low, sensitivity of molecular methods is variable generally below 70% at baseline.	LAMP use in treatment monitoring requires evaluation in the field.	91

Table 2: **Recommended testing strategies for detection of *T. cruzi* infections in different epidemiological and clinical settings.**

targeting the 196 bp Invariable Surface Glycoprotein gene, achieving a detection limit of 2.8×10^{-6} pg of DNA.[86] The visible results, obtained within 1 h, underscored colourimetric PSR as a practical, swift, sensitive, and specific tool for diagnosing Surra infections in livestock. This approach could be extended to *T. cruzi* diagnosis by employing corresponding molecular targets and primers.

CRISPR-CAS based strategies

CRISPR family members, such as Cas12, Cas13, and Cas14, demonstrate sequence-specific recognition and endonuclease activity known as CRISPR RNA (crRNA), featuring target-activated trans-cleavage. This enables the development of nucleic acid detection techniques. Sensitivity and specificity enhancement could be provided through the use of a fluorescent read-out and guide crDNA or crRNA, respectively. Trials with CRISPR-Cas13 (SHERLOCK)[87] and CRISPR-Cas12 (HOLMES: One-Hour Low-cost Multipurpose highly Efficient System)[88] on cutaneous leishmaniasis (CL)[89] and human African trypanosomiasis (HAT)[90] clinical samples targeted multi-copy genes (18S rDNA, kDNA minicircles) commonly used in trypanosomatid molecular diagnosis. While effective in nucleic acids detection, these methods require initial amplification (of RNA or DNA) through isothermal or traditional PCR, limiting their use in resource-limited regions.

Recommended diagnostic strategies for *T. cruzi* infection across diverse settings

Table 2 summarizes the primary testing strategies and algorithms recommended for diagnosing *T. cruzi* infection across various scenarios, including epidemiological surveys, blood donations, clinical diagnosis during acute and chronic infection phases, congenital CD diagnosis, detection of primary *T. cruzi* infection in seronegative recipients of organs from seropositive donors, identification of CD reactivation in immunosuppressed individuals with HIV coinfection or organ transplantation, and monitoring treatment response in clinical trials and healthcare settings.

Point-of-care (POC) diagnostics

CD prevails in tropical areas, mainly affecting impoverished communities. Consequently, the ideal test for

	Acceptable	Ideal
Sample type, volume and collection mode	Whole blood up to 100 µL collected by finger prick.	Whole blood up to 30 µL collected by finger prick.
Analytical specificity/ Exclusivity	Failure frequency of one every 1000 independent replicates/Does not cross-react with antibodies against other *Trypanosoma* spp. or *Leishmania* spp. and other related pathogens.	Failure frequency of one every 5000 independent replicates/Does not cross-react with antibodies against other *Trypanosoma* spp. or *Leishmania* spp. and other related pathogens.
Clinical specificity	≥95%, equivalent to that of conventional serological tools.	Idem.
Inclusivity	Regionally adapted tests capable to detect predominant strain/s circulating.	Single universal test detects all DTUs.
Clinical sensitivity	≥90% in comparison to conventional serological tools (the current standard algorithm).	≥95% in comparison to conventional serological tools (the current standard algorithm).
Assay controls	An internal control designed to identify the existence of human IgG, thereby confirming the proper flow of the sample.	Idem.
External quality controls	Proficiency testing panels evaluated before starting implementation of a new assay in the laboratory and every two years thereafter.	Proficiency testing panels evaluated every year.
Facility	Primary level health centers, community health posts.	Primary level health centers, community health posts, outdoors.
Equipment	None.	None.
Operator, training time	Laboratory technician, three days training.	Laboratory technician, one day training.
Time to get results	1 h since collection of sample.	30 min since collection of sample.

Table 3: **Target product profile for Chagas disease Point-of-Care serological tools.**

screening of potential infections or confirmatory diagnostic purposes should be able to be conducted in peripheral health facilities or mobile labs at the village level, in simple infrastructure conditions and with non-invasive sample collection. Serological RDTs were conceived precisely for these conditions. Isothermal amplification of nucleic acids also has the potential to be used in resource-limited areas, as it does not require complex nucleic purification steps nor specific and expensive equipment and reagents to perform the reaction and read the results. Among extant isothermal amplification methods,[92] four described technologies have been applied to detect trypanosomatids infection: NASBA, RPA, LAMP, and recently PSR. Two have been applied to *T. cruzi* infection: LAMP and RPA.

Target product profiles (TPPs) for the use of RDTs and isothermal amplification assays as POC CD diagnostics are proposed in Table 3 and Table 4. Both types of tests align with the REASSURED criteria, meeting the requirements of real-time connectivity, ease

	Acceptable	Ideal
Sample type, volume and collection mode	Anticoagulated whole blood (fluid blood): up to 500 µL/Filter paper dried blood spot (DBS): up to 125 µL.	Anticoagulated whole blood: 30 µL/Filter paper 3–6 mm DBS punch.
DNA extraction	Rapid DNA extraction, a single replicate.	Idem.
Analytical sensitivity	1 parasite per mL (par/mL) fluid blood/20 par/mL DBS.	0.1–0.5 par/mL.
Analytical specificity/ Exclusivity	Failure frequency of one every 1000 independent replicates/Does not cross-react with targets from other *Trypanosoma* spp. or *Leishmania* spp. and other related pathogens.	Failure frequency of one every 5000 independent replicates/Does not cross-react with targets from other *Trypanosoma* spp. or *Leishmania* spp. and other related pathogens.
Clinical sensitivity	≥95%. More than any microscopy test and similar than that of real time PCR (rtPCR).	≥98%. More than any microscopy test and similar than that of rtPCR.
Clinical specificity	Equivalent to microscopy tests and rtPCR (i.e., ≥98%), higher than ELISA.	Idem.
Inclusivity	Single universal test detecting all DTUs.	Idem.
Assay controls	Positive control included in kits, non-template control plus negative DNA extraction control.	Idem.
External quality controls	Proficiency testing panels evaluated before starting implementation of a new assay in the laboratory, and every two years thereafter	Proficiency testing panels evaluated every year.
Facility	Low complexity—2^{ry} level hospital.	Low complexity—2^{ry} level hospital.
Equipment	Wide availability and low cost of instruments and consumables, flexible instruments depending on the setting.	Integration of portable batteries, solar energy, mobile reporting applications, and other components, resulting in the fulfillment of the REASSURED criteria.[93]
Operator, training time	Biochemist or related profession, five days training.	Biochemist or related profession, two days training.
Time to get results	5 h since collection of sample.	2.5 h since collection of sample.

Table 4: **Target product profile for Chagas disease Point-of-Care molecular tools.**

of specimen collection, affordability, sensitivity, specificity, user-friendliness, rapidity, equipment-free operation, and deliverability to end-users.[93]

The development of CD isothermal amplification products is an incipient area. Yet, there are already dozens of serological RDTs from various manufacturers in the market.[25] In that context, ensuring test quality in decentralised settings poses a significant challenge. This diversity may affect diagnostic accuracy, leading to inconsistent results across sites. To maintain testing quality, a reference laboratory should dispatch proficiency panels to all POC testing sites. Incorporating blinded samples with varying parasitic loads and non-infected controls in each control panel in every test box ensures the validation of the assays upon their arrival at the destination and prior to their initial use in the field. For example, a recent External Quality Control (EQC) assessment conducted on the *T. cruzi*-LAMP prototype by Eiken Chemical Inc. Ltd. (Tokyo, Japan) utilised proficiency testing panels with spiked blood samples collected from DBS in nine study sites.[84]

Another challenge for the adoption of CD POC tests is the establishment of data connectivity systems. The appropriate management of epidemiological data and data flows is a pressing need in most public health systems. Having such an integrative strategy would be crucial to ensure the communication of results, guarantee their quality and traceability, and activate alerts for corrective action if needed, while fostering a decentralized approach to diagnostics.

The need for novel diagnostic algorithms

In 2009, the WHO Department of NTDs initiated the formation of a monitoring and evaluation working group to develop standardized tools for NTD frameworks. This response to challenges in NTD programs, including unreliable diagnostic tools, led to the creation of the Diagnostics Technical Advisory Group (DTAG). As a collaborative platform, DTAG aligns with the 2030 roadmap, reviewing NTD program needs, defining use cases, and guiding new tool development for WHO's NTD control and elimination goals.[94] In 2015, the PAHO's expert panel outlined critical attributes for diagnostic methods in *T. cruzi* infection scenarios, creating a TPP.[91] This framework, covering aspects from medical conduct to technical skills, ensures accuracy and minimizes cross-diagnosis risks in individual care, population programs, situational contexts, and equipment specifications.[1,91]

The 2030 WHO roadmap aims to eliminate CD as a public health concern; however, the absence of a single suitable test presents a challenge. The current diagnostic algorithms, although accurate, rely on multiple tests and entail lengthy turnaround times, posing a challenge to patient follow-up, particularly in resource-limited settings.[52] Addressing this impracticality requires the development of innovative algorithms that can integrate RDTs, digital microscopy/cell phone imaging tools, isothermal amplification strategies, and automated screening tools for blood banks.

Improving healthcare accessibility, especially in peripheral areas, requires deploying portable diagnostic devices, training community health workers, utilising mobile health solutions, establishing telemedicine networks, investing in low-cost diagnostic technologies, fostering public-private partnerships, conducting capacity-building programs, addressing infrastructure challenges, and implementing robust data management systems. It is essential to incorporate these approaches to build a thorough and accessible diagnostic infrastructure, particularly in challenging environments. This initiative should be preceded by field research to ensure effective community implementation.

Search strategy and selection criteria

References for this review were identified through searches of PubMed with the search terms "Chagas disease", "*Trypanosoma cruzi*", "diagnosis", "parasitological", "serological", "molecular", "point-of-care", "LAMP", and "RDTs." Searches were conducted from the database inception until January, 2024. Articles were also identified through searches of the authors' own files including articles written in Spanish and Portuguese. The final reference list was generated based on originality and relevance to the broad scope of this review.

Final remarks

In addition to crafting academic reviews that consolidate emerging insights from new investigations and field studies, it is crucial to disseminate notable advancements in CD diagnosis. This dissemination must concurrently target policymakers, health economists, kit manufacturers, regulatory agencies and patients' associations to foster the integration of innovative tools into healthcare systems. A collaborative partnership with international organizations and networks is vital to exert influence on governments and urge them to take prompt action. Without such concerted efforts, the rapid evolution of emerging technologies may face delays in implementation, potentially rendering them outdated and obsolete.

Contributors
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We declare no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.lana.2024.100821.

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